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Application of: Bertrand Seraphin	Confirmation No. 5538
Application No.: 09/785,793	Group Art Unit: 1645
Filed: February 16, 2001	Examiner: Hines, Jana A
For: Method for Purifying Proteins and/or Biomolecule or Protein Complexes	Attorney Docket No.: 11621-004-999 (formerly: 70436)

DECLARATION OF DR. ULRICH KRUSE UNDER 37 C.F.R. § 1.132

I, Dr. Ulrich Kruse, of Dossenheim, Germany, do declare and state that:

1. I am currently employed as a Senior Scientist in the Department of Discovery Research at Cellzome AG in Heidelberg, Germany. My current job title is Scientific Coordinator Biology. Cellzome AG has exclusively licensed U.S. patent application no. 09/785,793 entitled "Method for Purifying Proteins and/or Biomolecule or Protein Complexes" (the "'793 application") from the assignee of the '793 application, the Europaisches Laboratorium Fur Molekularbiologie (EMBL). I carried out my Ph.D. project on the Nuclear Factor I family of transcription factors in the laboratory of A. E. Sippel at the Universities of Heidelberg and Freiburg and received a Ph.D. in Biology in 1992 (summa cum laude). This work was followed by postdoctoral studies in the laboratory of Peter K. Vogt at the University of Southern California, Los Angeles, and at the Scripps Research Institute, La Jolla. My projects on oncogenes and transcriptional regulation resulted in several publications. After a teaching position at the University of Freiburg, I worked for Evotec Biosystems, Hamburg, and since 2001, for Cellzome AG. My curriculum vitae

and a list of my publications are attached as Exhibit 1.

2. My scientific background comprises expertise in biochemistry and molecular biology. In my Diploma thesis I identified, cloned and purified bacteriophage T4-encoded proteins that associate with the host E. coli RNA polymerase. This project included the purification of enzymatically active RNA polymerase with conventional protein purification techniques including DNA-affinity chromatography. My Ph.D project was focused on the regulation of mammalian gene expression through transcription factors. The suite of experimental procedures comprised standard DNA cloning and sequencing, transfection of mammalian cell lines with expression vectors, reporter gene assays as well as DNA-protein interaction studies in vitro (gel shift, DNase footprinting). Since the NFI family of transcription factors forms stable homo- and heterodimers I gained experience in protein-protein interaction studies. During my postdoctoral projects I worked on nuclear oncogenes such as v-Jun using retrovirus-based mammalian expression systems.

3. At Cellzome AG, I headed a cancer research project that applied the "TAP method"[1] to purify and identify several protein complexes from human cells that are involved in the malignant transformation of human cells. This project resulted in the purification and identification of novel protein components of the telomerase complex.

4. I have reviewed the '793 application, the claims as presently pending and as proposed to be amended, and the Office Action dated July 15, 2005. I have been asked whether, in

[1] TAP is the abbreviation for Tandem Affinity Purification. Although the term "TAP method" is not mentioned in the '793 application, the scientific community has come to know this technique by this name due to the multiple affinity purification steps that are part of this method. For ease of reference, I will refer to the methods that are described and claimed in the '793 application collectively as the TAP method.

August 1998, a skilled scientist,[2] following the teachings and guidance set out in the '793 application, would be able, using only routine experimentation, (i) to apply the claimed methods of the '793 application to nucleic acids encoding different polypeptides of interest; (ii) to apply the claimed methods to eukaryotic expression systems other than yeast cells, such as mammalian expression systems; and (iii) to implement the claimed methods using affinity tags different from the ProtA-TEV-CBP double tag that was used in Example 1 of the '793 application. For reasons I explain in detail below, it is my opinion that a skilled scientist would be able to carry out (i) to (iii), above.

5. The '793 application[3] describes a method, the TAP method, for the purification of a polypeptide of interest, in association with other biomolecule(s) in a complex when such polypeptide participates in such a complex in a eukaryotic expression environment, from eukaryotic cells or eukaryotic *in vitro* expression systems. Applying the TAP method, a skilled scientist can use a polypeptide of interest as a bait to purify and subsequently characterize the biomolecular assemblies with which the polypeptide of interest is associated. The TAP method can be performed by following distinct steps: (i) generation of a nucleic acid that encodes a fusion protein of a polypeptide of interest fused to at least two different affinity tags (see paragraphs 0007; 0030 to 0034; 0044 to 0048; and 0051 of the '793 application); (ii) expression in a eukaryotic environment, either by recombinant production within a eukaryotic cell or by use of a eukaryotic cell-free expression system (see paragraphs 0022 to 0025; 0027; and 0054 of the '793 application); and (iii)

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[2] I believe that a skilled scientist with respect to the field of the '793 application would be proficient in the fields of molecular biology and/or protein biochemistry.

[3] Citations to the '793 application herein will be to the application as published under publication number US 2002/0061513.

purification of the fusion protein by two sequential affinity purifications using its affinity tags (see paragraphs 0028 to 0029 of the '793 application). In my opinion, each individual step requires merely the application of standard protocols that were commonly known and readily available to the skilled scientist in August of 1998, as discussed further below.

6. It is also my opinion that the TAP method is applicable to cellular polypeptides in general. By the late 1990's, it had become apparent that "nearly every major process in a cell is carried out by assemblies of 10 or more protein molecules" (Alberts, 1998, Cell 92:291-294, at p. 291, left col.; "Alberts;" attached hereto as Exhibit 2; see also Gavin et al., 2002, Nature 415:141-147; "Gavin;" attached as Exhibit 3). Because of the vast numbers of polypeptides in eukaryotic cells that are expected to be components of protein assemblies in the cell, it is not necessary to test whether the polypeptide of interest is part of a biomolecule complex before applying the TAP method. Rather, the skilled scientist could select a polypeptide of his or her choice and follow the procedure laid out and claimed in the '793 application, with a high probability of success in detecting and purifying the biomolecule complex with which the polypeptide of interest is associated, when such polypeptide is present in a complex in a eukaryotic expression environment. If the polypeptide does not participate in a complex in a eukaryotic expression environment, the method will purify the polypeptide alone.

7. In fact, it has been demonstrated that the TAP method is suitable for large scale analyses of polypeptides for a proteome-wide characterization of protein assemblies, showing that the method is widely applicable to cellular proteins. Gavin (Exhibit 3) applied the TAP technology to 1,739 open reading frames, *i.e.*, 1,739 polypeptides of interest, to gain

insight in protein-protein interactions on a proteome level. Starting with these 1,739 open reading frames, Gavin et al. purified 589 tagged fusion proteins, 78% of which were found to be associated in complexes, with 232 non-redundant protein complexes being identified. The success rates of the individual steps: generation of the fusion proteins, expression of the fusion proteins, TAP purification, and identification of the complexes are shown in Figure 1c at page 142 of Gavin. Remarkably, despite the high throughput approach, the success rate for each step is far above 50%. Thus, Gavin has demonstrated that the TAP technology is generally applicable to polypeptides, and that the TAP technology can be routinely applied to different polypeptides without specific adjustment of the TAP method to each individual polypeptide of interest.

8. In view of common knowledge in the fields of molecular biology and/or biochemistry in August 1998 and the description in the '793 application, the selection of suitable affinity tags would have been routine. The '793 application describes that the polypeptide of interest is fused to at least two different affinity tags. Several exemplary affinity tags are listed in the '793 application at paragraph 0007; many more affinity tags that can be used routinely were well-known in the art. In fact, affinity tags, their binding partners, and methods for purification of proteins using these affinity tags were standard techniques in August of 1998 (see, *e.g.*, Section 4 entitled "Protein Recognition Tags Based on Affinity Interactions," at pp. 10 to 17 of Jones *et al.*, 1995, J. Chromatography A 707:3-22; "Jones;" attached hereto as Exhibit 4). The '793 application teaches at paragraph 0038 that the purification steps are chosen such that biomolecule complexes are maintained. In August 1998, it was well-known to a skilled scientist that certain buffer conditions, such as for example high or low pH, presence of chaotropic agents, or harsh detergents, would

lead to dissociation of biomolecule complexes, and that buffers that tended to simulate physiological conditions (*e.g.*, of pH and salt concentration) would be preferred. The types of molecular interactions affecting the stability of individual proteins and the association of proteins with other proteins were well-known (hydrogen bonds, hydrophobic interactions, and ionic interactions); also well-known were conditions (*e.g.*, based on temperature, salt concentration, pH values) that would preserve these interactions (see, *e.g.*, Marshak et al., 1996. Strategies for Protein Purification and Characterization. A Laboratory Course Manual. Cold Spring Harbor Laboratory Press 1996, Introduction, pp. 1 to 10; attached as Exhibit 5). Accordingly, the skilled artisan would have chosen affinity tags that allow elution from the affinity matrix under conditions that would not dissociate the biomolecule complexes (*e.g.*, elution with chelating agent; see paragraph 0044 of the '793 application). Alternatively or additionally, the skilled scientist could separate the affinity tag from the polypeptide of interest by a cleavage site to allow release of the polypeptide (or the truncated fusion protein) as described at paragraphs 0029-0033 and 0048 of the '793 application. Such cleavage sites were also well-known to a skilled scientist. For example, Jones (Exhibit 4, in particular the paragraph spanning pp. 11 and 12), discusses cleavage sites that can be used to separate a polypeptide of interest from an affinity tag. Thus, it is my opinion that the skilled scientist could select and use various suitable combinations of affinity tags and cleavage site(s) to purify a particular biomolecule complex in a straightforward manner based on the teachings of the '793 application and standard knowledge in the field.

9. It is also my judgment that creating a recombinant nucleic acid containing a nucleotide

sequence that encodes the polypeptide of interest fused to two or more affinity tags with one or more optional cleavage sites intervening between the encoded tag and the encoded polypeptide could be performed using standard cloning techniques that were routine in August 1998. With regard to the location of the affinity tags, the '793 application teaches that the affinity tags can be fused at the N- or C-terminus of the polypeptide, or even internally in the polypeptide (paragraph 0047). Commonly known vectors and molecular cloning techniques that can be used, for example, are described in Sambrook et al. (1989, 2nd Edition, Strategies for Cloning in Plasmid Vectors, pp. 1.53 to 1.73, In: Molecular Cloning—A Laboratory Manual, Cold Spring Harbor Laboratory Press; attached as Exhibit 6), which is a standard textbook that is cited at paragraph 0050 of the '793 application.

10. As taught in the '793 application, where the skilled scientist chooses to express the fusion protein in a recombinant host cell, the following individual steps can also be part of the TAP method: (a) transfection of the nucleic acid that encodes the fusion protein into a host cell (paragraph 0023 of the '793 application); (b) expression of the nucleic acid in the host cell (paragraphs 0021 to 0025; and 0027 of the '793 application); and (c) extraction of proteins from the cell (paragraphs 0006 and 0025 of the '793 application). Transfection of nucleotide sequences into cells was also a very well established, routine technique in August 1998. Protocols for the transfection of nucleotide sequences into different cell types such as insect cells, yeast cells, and mammalian cells were standard in molecular biology laboratories at that time. For example, Sambrook et al. (1989, 2nd Edition, Introduction of Recombinant Vectors into Mammalian Cells, pp. 16.30 to 16.72, In: Molecular Cloning—A Laboratory Manual, Cold Spring Harbor Laboratory Press;

attached as Exhibit 7) provides protocols for transfection of DNA into mammalian cells.

11. Similarly, numerous protocols for the expression of a nucleotide sequence in a eukaryotic expression system were readily available to a skilled scientist in August 1998. Appropriate promoters for the expression system of choice were also standard tools. For example, Sambrook et al. (1989, 2nd Edition, Functional Components of Mammalian Expression Vectors, pp. 16.5 to 16.29, In: Molecular Cloning—A Laboratory Manual, Cold Spring Harbor Laboratory Press; attached as Exhibit 8) discusses promoters, enhancer sequences, and polyadenylation sites that can be used for recombinant expression of proteins in mammalian cells. Further, an overview of protein expression in mammalian cells is also provided in Ausubel et al. ((Editors), 1995, Unit 16.12: Overview of Protein Expression in Mammalian Cells, In: Short Protocols in Molecular Biology, Third Edition, John Wiley & Sons, Inc., attached hereto as Exhibit 9). The '793 application discloses at paragraph 0050, consistent with what was well known in the art of molecular biology and/or biochemistry, that the expression of the fusion protein is regulated by control sequences, such as promoters, enhancers, and poly-A sites. The '793 application also provides a working example using expression in yeast cells (see paragraphs 0060 to 0069). Furnished with the teachings of the '793 application and the general knowledge in the field of molecular biology in August 1998, a skilled scientist could have routinely selected suitable control sequences, vectors, and host cells for the expression of the fusion protein in eukaryotic cells. Alternatively, use of a cell-free system to achieve a eukaryotic expression environment was also well-known in the art (see, *e.g.*, King et al., 1997, Science 277:973-974; attached hereto as Exhibit 10; see also paragraph 0027 of the '793 application).

12. Obtaining protein extracts from cells and manipulation of cell extracts under conditions that leave many protein and other biomolecule complexes intact has been a standard technique in biology laboratories for a long time, and was well-established by August 1998. For example, Smith (May 1998, Methods 15(1):27-39; attached hereto as Exhibit 11) published a protocol for the preparation of cell extracts in order to study RNA editing protein complexes assembled on Apolipoprotein A mRNA. This extraction protocol was successfully applied to diverse cell types such as McArdle 7777 rat hepatoma, HepG2 human hepatoma, HeLa human cervical carcinoma, Chinese hamster ovary (CHO), and a variety of other mammalian cells (see especially pp. 30-32 of Exhibit 11). As discussed above, conditions that would not dissociate biomolecule complexes were well-known to a skilled scientist. In view of the state of the art and the direction in the '793 application to maintain the biomolecule complexes (paragraph 0038), a skilled scientist would have been taught, and able routinely, to avoid harsh conditions that would be expected to dissociate biomolecule complexes.

13. The '793 application teaches that the fusion protein of interest, and with it the protein complex of which the fusion protein is a part, is purified by virtue of its affinity tags. The guidance for the affinity purification in the '793 application and the state of the art of affinity purification in August 1998 are discussed above in paragraph 8, showing that only routine skill would be expected to be needed in the implementation of those steps.

14. The '793 application teaches that the TAP method is applicable to eukaryotic cells in general. Example 1 in the '793 application and Gavin (Exhibit 3) provide evidence that the TAP method can be successfully used for polypeptides in yeast. Additional evidence demonstrates that the TAP method is applicable to other eukaryotic cells, such as

mammalian cells. For example, Cox et al. (2002, Biotechniques 33:267-270; "Cox;" attached hereto as Exhibit 12) shows the successful application of the TAP method in a mammalian expression system. Following the steps of the TAP method, Cox purified a protein complex from mammalian cells. For each individual step of the TAP method, a skilled scientist, using merely routine experimentation, could have adopted the guidance of the '793 application and arrived at the specific conditions used in Cox. I discuss this in detail in the following paragraphs.

15. Cox used an N-terminal TAP affinity tag. Cox's fusion protein included the following elements in the following order starting at the N-terminus: two IgG binding domains of *Staphylococcus aureus* protein A ("ProtA"), TEV protease cleavage site ("TEV"), calmodulin binding site ("CBP"), enterokinase cleavage site ("EK"), and Cox's polypeptide of interest (MEF2-A).[4] Apart from the second cleavage site (EK), Cox used the same CBP-TEV-ProtA double tag that is described in the '793 application (see paragraph 0048). In fact, Cox obtained the TAP tag vectors from Dr. Seraphin, an inventor of the '793 application (see Acknowledgment at page 269 of Cox). That a second cleavage site can be part of the TAP tag is disclosed in the '793 application at paragraph 0048.

16. Cox used a commercially available expression vector with a cytomegalovirus (CMV) promoter and tetracycline-regulated control sequence. Both regulatory sequences were commonly known and routinely used by skilled scientists in August 1998 for expression

[4] Although the individual elements of the fusion protein are not shown in Figure 1 of Cox, at p. 268, left col., it is stated that the "TAP tag was excised from the yeast vector pBS-1761." As a reference for pBS-1761, Puig et al., 2001, Methods 24:218-229 is cited ("Puig;" attached hereto as Exhibit 13). The components of the TAP tag that is encoded by a nucleotide sequence that is part of the pBS-1761 vector are shown in Figures 1 and 2 of Puig.

of a protein of interest in a mammalian cell (see, *e.g.*, Holwell et al., 1997, J Cell Science 110:1947-1956; attached hereto as Exhibit 14; see also Sambrook (Exhibit 8)). Many other promoters that can be used for expression in mammalian cells such as the COS7 cells used by Cox were well-known in August 1998 (see, *e.g.*, Sambrook (Exhibit 8)). The TAP tag, *i.e.*, ProtA-TEV-CBP-EK and the coding region for MEF2-A were cloned into the cloning vector using standard molecular cloning techniques.

17. Cox transfected the expression vector into the mammalian COS7 cell line using calcium phosphate precipitation. Calcium phosphate precipitation was a standard technique to transfect eukaryotic cells with DNA as of August 1998 (see, *e.g.*, Exhibit 7).

18. Protein extract was obtained from the transfected cells in Cox by lysing the cells by freeze/thaw cycles. After lysis, Nonidet P-40 (a nonionic detergent) was added. Both the use of NP-40 and freeze/thaw cycles were routine in the art (see, *e.g.*, Sambrook et al. (1989, 2nd Edition, Immunoprecipitation, pp. 18.26 to 18.33, In: Molecular Cloning—A Laboratory Manual, Cold Spring Harbor Laboratory Press; attached as Exhibit 15; and Chapter 16 Expression of Cloned Genes in Cultured Mammalian Cells, pp. 16.59 to 16.62; attached as Exhibit 16), and are not harsh conditions that generally dissociate protein-protein complexes. Since the '793 application teaches that protein complexes, where present, are to be isolated (see, *e.g.*, paragraph 0038 of the '793 application), it is my opinion that a skilled scientist would know to choose conditions such as these that minimize the dissociation of protein-protein interactions.

19. Cox also used routine experimentation and methods taught in the '793 application to conduct the affinity purification steps. Both the ProtA and the CBP affinity purifications used by Cox are taught in the '793 application, and commonly known in the art (see, *e.g.*,

Jones (Exhibit 4)). Again, since protein complexes were to be purified, a skilled scientist would have been aware that conditions that generally would dissociate biomolecule complexes are to be avoided.

20. Using procedures as described in the '793 application in Example 1 (paragraphs 0060 to 0068), Cox purified the complex via binding to a support, cleavage with Tobacco Etch Virus protease NIA, binding to a support, and elution, all using essentially the same method as described in the '793 application, thereby evidencing the straightforward applicability of the TAP method to mammalian cells.

21. Further evidence of the straightforward applicability of the TAP method to mammalian cells is found in Ju et al. (2004, Cell 119:815-829; "Ju;" attached hereto as Exhibit 17). Ju provides another example of the application of the TAP method to mammalian cells, specifically to 293 cells. In the Experimental Procedures section, Ju relies on a reference to Puig (Exhibit 13), which essentially describes the TAP method as taught in the '793 application.

22. In view of the foregoing facts and analysis, I have concluded that, in August 1998, a skilled scientist, using only routine experimentation, would have been able (i) to apply the claimed methods of the '793 application for purifying polypeptides, or biomolecule complexes containing polypeptides, from a eukaryotic expression system in general, such as a mammalian or yeast system; (ii) to apply these methods to nucleic acids encoding different polypeptides of interest; and (iii) to implement these methods using different affinity tags.

23. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that

these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

January 13, 2006

DATE

DR. ULRICH KRUSE

Attachments:

Exhibit 1	Curriculum vitae of Dr. Ulrich Kruse with list of publications
Exhibit 2	Alberts, 1998, Cell 92:291-294
Exhibit 3	Gavin et al., 2002, Nature 415:141-147
Exhibit 4	Jones et al., 1995, J. Chromatography A 707:3-22
Exhibit 5	Marshak et al., 1996, Introduction, pp. 1 to 10, In: Strategies for Protein Purification and Characterization—A Laboratory Course Manual, Cold Spring Harbor Laboratory Press
Exhibit 6	Sambrook et al., 1989, 2nd Edition, Strategies for Cloning in Plasmid Vectors, pp. 1.53 to 1.73, In: Molecular Cloning—A Laboratory Manual, Cold Spring Harbor Laboratory Press
Exhibit 7	Sambrook et al., 1989, 2nd Edition, Introduction of Recombinant Vectors into Mammalian Cells, pp. 16.30 to 16.72, In: Molecular Cloning—A Laboratory Manual, Cold Spring Harbor Laboratory Press
Exhibit 8	Sambrook et al., 1989, 2nd Edition, Functional Components of Mammalian Expression Vectors, pp. 16.5 to 16.29, In: Molecular Cloning—A Laboratory Manual, Cold Spring Harbor Laboratory Press
Exhibit 9	Ausubel et al. ((Editors), 1995, Unit 16.12: Overview of Protein Expression in Mammalian Cells, In: Short Protocols in Molecular Biology, Third Edition, John Wiley & Sons, Inc.
Exhibit 10	King et al., 1997, Science 277:973-974
Exhibit 11	Smith, 1998, Methods 15(1):27-39
Exhibit 12	Cox et al., 2002, Biotechniques 33:267-270

Exhibit 13	Puig et al., 2001, Methods 24:218-229
Exhibit 14	Holwell et al., 1997, J Cell Science 110:1947-1956
Exhibit 15	Sambrook et al., 1989, 2nd Edition, Immunoprecipitation, pp. 18.26 to 18.33, In: Molecular Cloning—A Laboratory Manual, Cold Spring Harbor Laboratory Press
Exhibit 16	Sambrook et al., 1989, 2nd Edition, Expression of Cloned Genes in Cultured Mammalian Cells, pp. 16.59 to 16.62, In: Molecular Cloning—A Laboratory Manual, Cold Spring Harbor Laboratory Press
Exhibit 17	Ju et al., 2004, Cell 119:815-829